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Corrêa Pereira, Marlon; Moreira Vieira, Nívea; Tótola, Marcos Rogério; Megumi Kasuya, Maria Catarina

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TOTAL FATTY ACID COMPOSITION IN THE CHARACTERIZATION AND IDENTIFICATION OF ORCHID MYCORRHIZAL FUNGI *Epulorhiza* spp.[1]

Marlon Corrêa Pereira[2], Nívea Moreira Vieira[3], Marcos Rogério Tótola[4] & Maria Catarina Megumi Kasuya[4]

SUMMARY

***Rhizoctonia*-like fungi are the main mycorrhizal fungi in orchid roots. Morphological characterization and analysis of conserved sequences of genomic DNA are frequently employed in the identification and study of fungi diversity. However, phytopathogenic *Rhizoctonia*-like fungi have been reliably and accurately characterized and identified through the examination of the fatty acid composition. To evaluate the efficacy of fatty acid composition in characterizing and identifying *Rhizoctonia*-like mycorrhizal fungi in orchids, three *Epulorhiza* spp. mycorrhizal fungi from *Epidendrum secundum*, two unidentified fungi isolated from *Epidendrum denticulatum*, and a phytopathogenic fungus, *Ceratorhiza* sp. AGC, were grouped based on the profile of their fatty acids, which was assessed by the Euclidian and Mahalanobis distances and the UPGMA method. Dendrograms distinguished the phytopathogenical isolate of *Ceratorhiza* sp. AGC from the mycorrhizal fungi studied. The symbionts of *E. secundum* were grouped into two clades, one containing *Epulorhiza* sp.1 isolates and the other the *Epulorhiza* sp.2 isolate. The similarity between the symbionts of *E. denticulatum* and *Epulorhiza* spp. fungi suggests that symbionts found in *E. denticulatum* may be identified as *Epulorhiza*. These results were corroborated by the analysis of the rDNA ITS region. The dendrogram constructed based on the Mahalanobis distance differentiated the clades most clearly. Fatty acid composition analysis proved to be a useful tool for characterizing and identifying *Rhizoctonia*-like mycorrhizal fungi.**

Index terms: *Rhizoctonia*-like fungi, *Epidendrum* spp. orchids, FAME, MIDI method, ITS sequence analysis.

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[2] Professor of Biological Science and Health Institute, UFV, Campus of Rio Paranaíba. MG 230, Km 7, Caixa Postal 22, CEP 38810-000 Rio Paranaíba (MG), Brazil. E-mail: marlon.pereira@ufv.br

[3] Technique of Plant Anatomy Laboratory, Plant Biology Department, UFV. Av. Peter Henry Rolfs, s/n, CEP 36570-000 Viçosa (MG), Brazil. E-mail: nimvieira@gmail.com

[4] Professors of Microbiology Department, UFV. Av. Peter Henry Rolfs s/n, CEP 36570-000 Viçosa (MG), Brazil. E-mails: totola@ufv.br; mkasuya@ufv.br

RESUMO: *COMPOSIÇÃO DE ÁCIDOS GRAXOS NA CARACTERIZAÇÃO E IDENTIFICAÇÃO DE FUNGOS* Epulorhiza spp. *MICORRÍZICOS DE ORQUÍDEA*

Os fungos rizoctonioides são os principais fungos micorrízicos de orquídeas. A caracterização morfológica e a análise de sequências conservadas do DNA total são as principais estratégias empregadas na identificação e no estudo da diversidade desses fungos. Contudo, fungos rizoctonioides fitopatogênicos têm sido caracterizados e identificados, de forma confiável e precisa, pela determinação da composição de seus ácidos graxos. Com o objetivo de avaliar a utilização da composição de ácidos graxos na caracterização e identificação de fungos rizoctonioides micorrízicos de orquídeas, três fungos Epulorhiza *spp., micorrízicos de* Epidendrum secundum*, dois fungos não identificados, isolados de* Epidendrum denticulatum*, e um fungo fitopatogênico* Ceratorhiza *sp. AGC foram agrupados com base no perfil de seus ácidos graxos, utilizando as distâncias euclidiana e de Mahalanobis e o método UPGMA. Os dendrogramas gerados possibilitaram a distinção entre o isolado fitopatogênico de* Ceratorhiza *sp. AGC e os fungos micorrízicos estudados. Os simbiontes de* E. secundum *foram distribuídos em dois clados: o primeiro contendo os isolados de* Epulorhiza *sp.1, e o segundo, o isolado de* Epulorhiza *sp.2. A similaridade entre os simbiontes de* E. denticulatum *e os fungos* Epulorhiza *spp. sugere a identificação desses simbiontes como* Epulorhiza *sp. Esses resultados foram semelhantes aos obtidos pela análise da região ITS (Internal Transcribed Spacer) do rDNA. O dendrograma construído a partir da distância de Mahalanobis apresentou melhor resolução, distinguindo claramente os clados descritos. A análise da composição de ácidos graxos mostrou ser uma ferramenta útil na caracterização e identificação dos fungos micorrízicos rizoctonioides estudados.*

Termos de indexação: fungos rizoctonioides, orquídeas Epidendrum spp.*, FAME, método MIDI, análise da sequência da região ITS.*

INTRODUCTION

The mycorrhizal fungi in orchids are primarily basidiomycetes of *Rhizoctonia*-like groups (Dearnaley, 2007; Pereira et al., 2005a). The main anamorph/ teleomorph genera observed in association with orchids are *Ceratorhiza* R.T. Moore/ *Ceratobasidium* D.P. Rogers, *Epulorhiza* R.T. Moore emend Andersen & R.T. Moore/ *Tulasnella* Schröeter, *Moliniopsis* Ruhland. (Sin. = *Rhizoctonia* D.C.)/ *Thanatephorus* Donk. (Sin. = *Botryobasidium* Donk p.p.), and *Opadorhiza* Andersen & R.T. Moore/ *Sebacina* Tul. (Currah & Zelmer, 1992; Andersen & Rasmussen, 1996; Taylor et al., 2002; Dearnaley, 2007; Pereira et al., 2005a,b, 2009). They are characterized by hyphae constriction in the septum, right-angle branching hyphae immediately after the septum, swollen cells, monilioid cells, sclerotia formation, a complex dolipore septum, absence of asexual spores, and a rare, difficult to observe sexual cycle (Stalpers & Andersen, 1996; García et al., 2006).

Analyses of morphological characteristics and conserved genomic sequences are frequently used in characterization and identification studies of *Rhizoctonia*-like mycorrhizal fungi from orchids (Rasmussen, 2002; Taylor et al., 2002; García et al., 2006; Dearnaley, 2007; Pereira et al., 2009).

Morphological characteristics are important in classical taxonomy to distinguish genera and species (Currah & Zelmer, 1992; Currah et al., 1997). Data obtained from these characteristics, when analyzed by biometric techniques, can be transformed into geometric distances to form clusters of the studied fungi (Cruz, 2008; Pereira et al., 2005b, 2009). In addition to identification and characterization, this type of analysis is useful for the assessment of the fungal diversity (Pereira et al., 2005b, 2009). However, the determination of some of these characteristics is time- and labor-intensive, calling for the use of quicker molecular methods with fewer and shorter phases (Taylor et al., 2002).

The analysis of conserved sequences of total DNA is used preferentially in the study of fungal symbionts isolated from orchids (Rasmussen, 2002; Taylor et al., 2002; Dearnaley, 2007). ITS region sequences of various mycorrhizal fungi of orchids are available in international databases, which allow the accurate identification of new isolates (Sharon et al., 2008).

The analysis of fatty acid composition is a quick and precise strategy used in the study of phytopathogenic *Rhizoctonia*-like fungi (Sharon et al., 2006). Different groups, species, and varieties of *Rhizoctonia*-like fungi have been characterized, differentiated, and identified based on their fatty acid profiles (Johnk & Jones, 1992, 1993, 1994; Johnk et al., 1993; Baird et al., 2000; Priyatmojo et al., 2002a; Lanoiselet et al., 2005). However, current literature reveals that fatty acid composition has not been used

for comparing and differentiating *Rhizoctonia*-like mycorrhizal fungi of orchids.

The aim of this study was to characterize *Rhizoctonia*-like mycorrhizal fungi from orchids, based on their fatty acid composition, and compare the fatty acid profiles of these fungi with results of ITS region sequence analyses.

MATERIAL AND METHODS

Five *Rhizoctonia*-like mycorrhizal fungi isolates were selected from orchids. Three isolates were obtained from *Epidendrum secundum* Lindl (Table 1). These isolates, identified as *Epulorhiza* sp. (Pereira et al., 2009), belong to a Collection of Orchid Mycorrhizal Fungi of the Laboratory of Mycorrhizal Associations, of the Biotechnology Institute for Agriculture (BIOAGRO) of the Federal University of Viçosa (UFV). The other two fungal isolates were obtained from *Epidendrum denticulatum* Barb. Rodr. (Table 1) by a procedure described by Pereira et al. (2009). For comparison, an isolate of the phytopathogenic fungus *Ceratorhiza* sp. AGC (Table 1) from the collection of phytopathogenic fungi of the department of phytopathology/UFV was also examined.

The fungi were grown in Petri dishes containing 25 mL of PDA medium (Potato Dextrose Agar, ACUMEDIA) and incubated at 28 °C in the dark for three days. Each isolate was grown in test tubes containing 1 mL of PDB (Potato Dextrose Broth, ACUMEDIA) inoculated with a 9 mm diameter disc containing active mycelium, with three replicates per isolate. After four days of incubation in the dark without agitation at 28 °C, mycelium samples were frozen at -86 °C, lyophilized and stored at -86 °C.

The Microbial Identification System (MIS, Microbial ID Inc., Newark, Delaware) was used to analyze the fatty acid composition of the samples. The methyl esters from fatty acids were obtained according to the protocol established for the MIDI method (Microbial Identification System, Microbial ID Inc., Newark, Delaware). Methyl esters were identified and quantified by gas chromatography, using Sherlock software (MIDI Inc., Newark, Delaware; version 4.5). Data on the proportions of fatty acids were arcsine square root-transformed and subjected to an analysis of variance (ANOVA) and means comparison by the Scott Knott test at 5 % significance, using Genes software, version 2007.0.0 (Cruz, 2008). Data were analyzed by the Standardized Euclidean and Mahalanobis distances. The calculated matrix distance was used for clustering analysis using the UPGMA method.

For DNA extraction, isolate mycelium was grown in 50 mL Erlenmeyer flasks containing 10 mL PDB medium, transferred to 1.5 mL Eppendorf tubes, frozen at -86 °C, and lyophilized. The lyophilized mycelium was used for total DNA extraction as described by Schäfer & Wöstemeyer (1992). The ITS (Internal Transcribed Spacer) region of nuclear rDNA was PCR-amplified using ITS1 and ITS4 oligonucleotides (White et al., 1990) under reaction conditions described by Gardes & Bruns (1993). The PCR products were purified using Exo-SAP (USB Corporation, Cleveland, Ohio), as recommended by the manufacturer. These products were sent to Macrogen Inc. (Geumchun-gu, South Korea) for automated cycle sequencing of both strands using BigDye TM and Automatic Sequencer 3730xl (Applied Biosystems).

The amplified ITS region sequences from the isolates were analyzed using the Sequencher software, version 4.5 (Gene Codes), for editing and construction of contigs. To classify the ED5.1A and ED5.3A isolates based on identity with fungal sequences deposited in the database of the National Center for Biotechnology Information - NCBI (GenBank, http://www.ncbi.nlm.nih.gov/), the contigs were subjected to BLAST analysis using the BlastN algorithm (Altschul et al., 1997). The contigs were also used to identify sequences of other fungi, *Ceratorhiza* / *Ceratobasidium* and *Epulorhiza* / *Tulasnella,* to perform the analysis based on the ITS region of rDNA. Sequences were aligned and analyzed using MEGA software version 4 (Tamura et al., 2007). The Neighbor-Joining method and the Kimura 2-Parameter nucleotide analysis model were used with 5000 bootstrap replicates, excluding gaps and missing data (Sharon et al., 2008). The sequences of the

Table 1. Isolate codes, identification of fungal species, host species, and origin of the *Rhizoctonia*-like fungi studied

Isolates	Identification	Host	Origin
AGC	*Ceratorhiza* sp.	-	-
ES2.3B	*Epulorhiza* sp.1	*E. secundum*	PESB/Serra das Cabeças, Araponga/MG
ES3.1A	*Epulorhiza* sp.2	*E. secundum*	PESB/Serra da s Cabeças, Araponga/MG
ES4.1M	*Epulorhiza* sp.1	*E. secundum*	PESB/Serra das Cabeças, Araponga/MG
ED5.1A	-	*E. denticulatum*	Restinga da Morada do Sol, Vila Velha/ES
ED5.3A	-	*E. denticulatum*	Restinga da Morada do Sol, Vila Velha/ES

studied fungi were deposited in NCBI with the accession numbers HQ127084 and HQ12708.

RESULTS

Eleven fatty acids were detected in the six isolates of the studied *Rhizoctonia*-like fungi. Only five were common to all isolates (Table 2). The analysis of variance showed significant differences among isolates for the proportion of each fatty acid detected, which allowed clustering of isolates based on each fatty acid by the Scott Knott test at 5 % significance (Table 2). In the grouping based on the Standardized Euclidian (D) and Mahalanobis (D2) distances and the UPGMA method, the proportion of fatty acids differentiated the *Ceratorhiza* sp. AGC isolate from *Epulorhiza* spp. isolates and between the two species of previously identified *Epulorhiza* (Figures 1 and 2). In the grouping by Mahalanobis distance, *E. denticulatum* isolates were clustered in a separate clade from the *E. Secundum* and *Epulorhiza* sp.1 isolates (Figure 2), a similar result to that obtained by ITS region analysis (Figure 3). The ITS region sequence distinguished *E. denticulatum* from other isolates of the *Epulorhiza* genus, which confirmed that *E. denticulatum* isolates belong to the *Epulorhiza* genus (Figures 3 and 4).

Table 2. Proportions of fatty acids detected in *Rhizoctonia*-like fungi isolates

Isolates	Fatty acid										
	14:00	Sum in feature 3	16:00	17:1 ω8c	17:0	16:0 2OH	18:3 ω6c (6,9,12)	Sum in feature 5	18:1 ω9c	18:00	20:00
AGC	1.01 a	1.47 c	11.66 a	0.47 d	0 b	0.58 a	0 b	82.86 a	0 b	1.95 b	0 b
ES2.3B	0 c	4.70 b	5.51 d	0.97 c	0 b	0 b	0 b	55.99 b	30.73 a	2.11 b	0 b
ES3.1A	0.51 b	5.56 b	9.17 b	1.33 b	0.33 a	0 b	0 b	44.95 c	34.12 a	3.71 a	0.36 a
ES4.1M	0.29 b	5.64 b	7.05 c	1.86 a	0 b	0 b	0 b	47.86 c	36.06 a	1.25 b	0 b
ED5.1A	0 c	13.15 a	8.67 b	0.94 c	0 b	0 b	0 b	41.77 d	33.06 a	2.41 b	0 b
ED5.3A	0.31 b	11.59 a	9.00 b	0.57 d	0 b	0 b	0.27 a	41.75 d	34.86 a	1.66 b	0 b
CV (%)	39.65	7.60	3.23	8.61	33.83	11.06	28.91	2.53	6.77	11.07	13.90

Data subjected to analysis of variance and the Scott Knott mean test at 5 % significance level, obtained after an arcsine square root-transformation of the proportions. Means in the columns followed by the same letter are statistically equal to each other. CV %, coefficient of variation; sum in feature 3 corresponds to 16:1 ω7c, or 15 iso2OH, or both; sum in feature 5 corresponds to 18 ANTE or 18:2 ω6, 9c, or both. Codes in the clusters correspond to the isolates presented in Table 1.

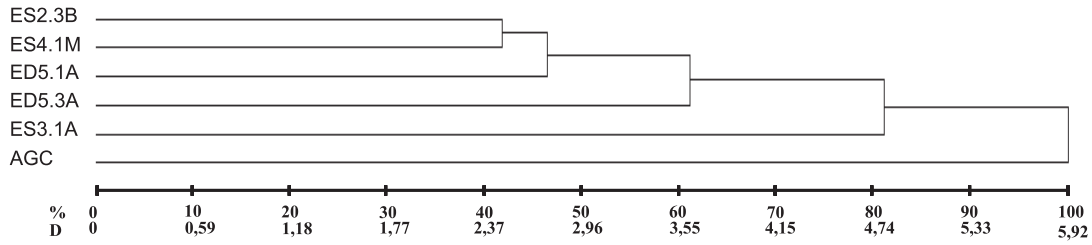

Figure 1. Clustering of *Rhizoctonia*-like fungi based on the averages of fatty acid proportions using the Standardized Euclidean Distance (D) and the UPGMA method. Codes in the clusters correspond to the isolates presented in Table 1.

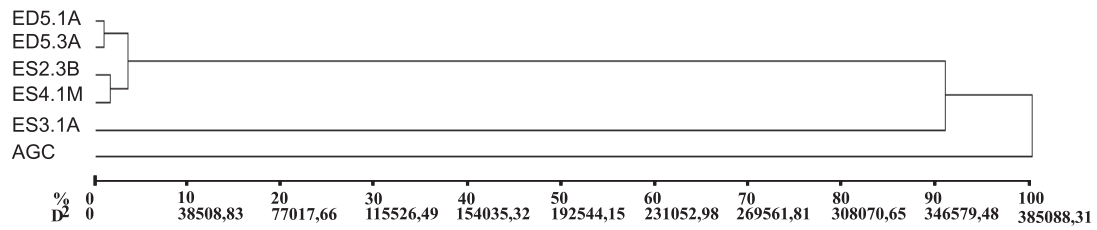

Figure 2. Clustering of *Rhizoctonia*-like fungi based on the proportions of fatty acids using the distance of Mahalanobis (D2) and UPGMA method. Codes in the clusters correspond to the isolates presented in Table 1.

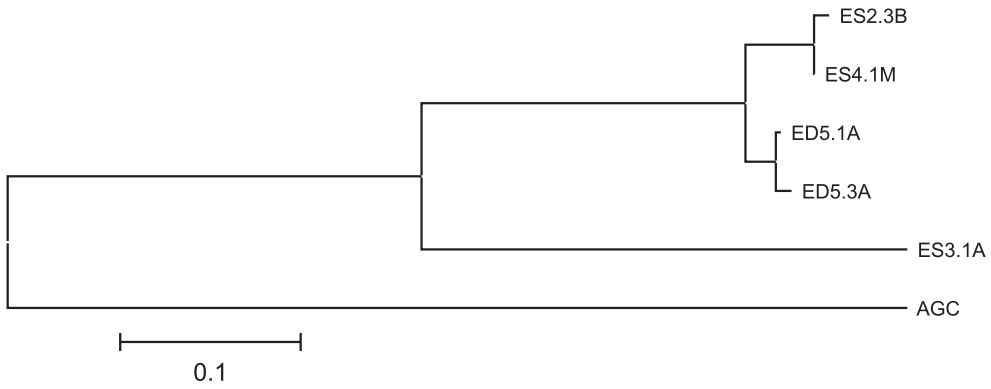

Figure 3. Clustering of *Rhizoctonia*-like fungi isolates based on rDNA sequences of the ITS region using the Neighbor-Joining method and distance matrix of the Kimura 2-parameter. Codes in the clusters correspond to the isolates presented in Table 1.

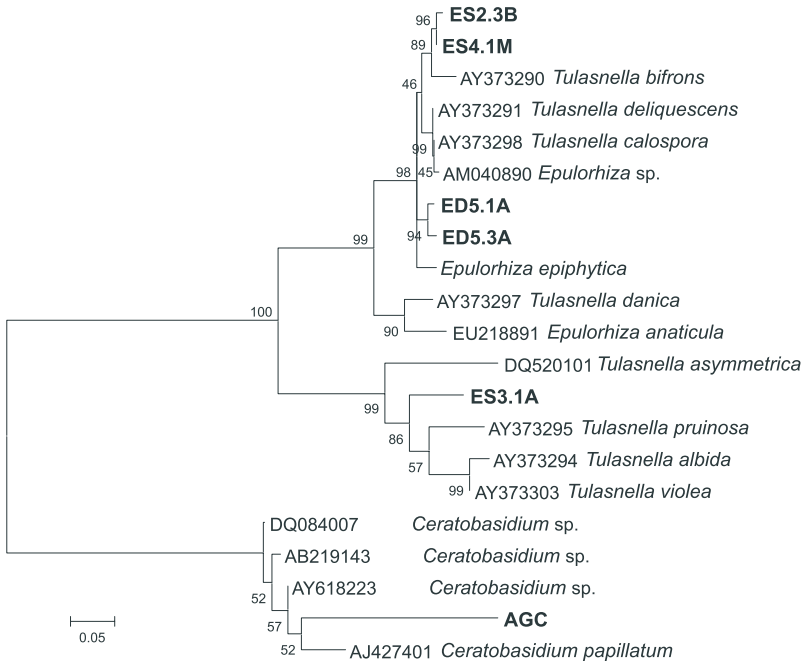

Figure 4. Clustering of *Rhizoctonia*-like fungi isolates and *Tulasnella* spp., *Epulorhiza* spp., and *Ceratobasidium* spp. fungi based on rDNA sequences of the ITS region using the Neighbor-Joining method and Kimura 2-parameter algorithm, based on 5000 bootstrap replicates, excluding gaps and missing data. Codes highlighted in the clusters correspond to the isolates presented in Table 1.

DISCUSSION

The fatty acid composition was used to characterize *Epulorhiza* spp. mycorrhizal fungi of orchids for the first time. The analysis of the fatty acid composition, in the detection of variability between different populations and varieties of the same species of phytopathogenic *Rhizoctonia*-like fungi (Baird et al., 2000; Priyatmojo et al., 2002a; Lanoiselet et al., 2005; Sharon et al., 2006), allowed the distinction of *Ceratorhiza* from *Epulorhiza* genera and among different *Epulorhiza* species (Figures 1 and 2).

The presence and/or absence of several fatty acids distinguished some groups of *Rhizoctonia*-like fungi. The fatty acids 18:1 ω9c were only present in *Epulorhiza* spp isolates, whereas 16:0 2OH were only detected in *Ceratorhiza* sp. AGC, and fatty acids 17:00 and 20:00 observed only in *Epulorhiza* sp.2 (Table 2). This suggests that these fatty acids may be used as analytical markers of isolates in pure culture. However, further studies are needed to confirm the use of fatty acids 18:1 ω9c, 16:0 2OH, 17:00, and 20:00 as markers for *Rhizoctonia*-like fungi of orchids.

The proportion of the other fatty acids must also be taken into account because, regardless of differences in the presence or absence of a particular fatty acid, some isolates can be grouped based on the total fatty acid composition. The presence of the 18:3 ω6c fatty acid (6,9,12) was only observed in the ED5.3A isolate, which was grouped with ED5.1A that was also obtained from *E. denticulatum* (Table 2, Figures 1 and 2). Another example are the three *E. secundum* isolates, with equal proportions of sum in feature 3, but which actually represent two different species that were separated in the final fatty acid composition analysis (Figures 1 and 2).

The clusters based on fatty acid composition and ITS region analysis were consistent (Figures 1, 2, and 3). This reinforces the use of the MIDI method of extraction, detection, and quantification of fatty acids for fungal characterization and in the identification of *Rhizoctonia*-like species (Lanoiselet et al., 2005; Sharon et al., 2006). Variability in the fatty acid composition was observed among *Epulorhiza* sp.1 and *E. denticulatum* isolates (Figures 1 and 2), which may be due to intra-specific variations, justifying its application in variability analyses within populations, as suggested by Sharon et al. (2006).

Even though the Standardized Euclidean distance is the most commonly used to quantitatively analyze fatty acid composition data (Baird et al., 2000; Priyatmojo et al., 2002a; Lanoiselet et al., 2005), the Mahalanobis distance increases the resolution of the analysis compared to clusters generated by other methods for calculating distances. The Standardized Euclidean distance was not able to distinguish between *E. denticulatum* mycorrhizal fungi and *Epulorhiza* sp.1 symbionts of *E. secundum* (Figure 1, Table 1). A possible reason is that the calculation of the Mahalanobis distance includeds residual variance and covariance, correcting distortions in the analysis (Cruz & Regazzi, 1997; Cruz, 2008), which are due to the high correlation among traits, i.e., the detected fatty acids. Different multivariate techniques must be used for data analysis for a validation of the observed results.

Fatty acid composition analysis is a promising tool in the characterization of fungi isolates of orchids, validated by the MIDI method, which proved to be sensitive to variations between fungi of the same species and different species and genera of *Rhizoctonia*-like fungi (Figures 2 and 3). As also observed for other *Rhizoctonia*-like fungi by this technique (Baird et al., 2000; Lanoiselet et al., 2005; Sharon et al., 2006). The storage of fatty acid profiles of fungi in the MIDI identification system can contribute to a library of *Rhizoctonia*-like fungi, with a view to use this technique in the characterization, identification, and analysis of the diversity of mycorrhizal fungi of orchids.

CONCLUSIONS

1. The analysis of the fatty acid composition distinguishes different species and different genera of *Rhizoctonia*-like fungi.

2. The resolution of the analyzed data is greater when the Mahalanobis distance is used for isolater grouping, based on the fatty acid composition, which is consistent with clustering based on ITS region sequence analysis.

3. Fatty acid composition analysis is a useful tool in the identification, characterization, and study of the diversity of mycorrhizal fungi of orchids.

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